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Recommendation for the unit of measurement and the measuring system to report traceable and comparable results expressing GM content in accordance with EU legislation

Corbisier P., Barbante A., Berben G., Broothaerts W., De Loose M., Emons H., Georgieva Tz, Lievens A., Mazzara M., Papazova N., Perri E., Sowa S., Stebih D., Terzi V., Trapmann S.

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Contact information
Name: Philippe Corbisier
Address: Retieseweg 111, B-2440 Geel
Email: philippe.corbisier@ec.europa.eu
Tel.: +32 14 571 890

JRC Science Hub
https://ec.europa.eu/jrc

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Recommendation for the unit of measurement and the measuring system to report traceable and comparable results expressing GM content in accordance with EU legislation

Contributors:

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1 Abstract

It is important to guarantee that results expressing the GM content are reliable, comparable and fulfil the requirements of existing EU legislation. The use of different measurement units to express a GM content, the appearance of new analytical methods that do not require a calibrant and the composite EU legislation on GMOs have triggered the need for a document to clarify how to obtain reliable and comparable results.

For this guidance document, past and current EU legislations have been reviewed with a special emphasis on what is meant by 'GM percentage' in the different legal texts. The metrological traceability of measurement results and the currently available guidance are explained and summarised. The particular case of botanical impurities and the genetic constitution of GM seeds are described and illustrated to better understand the complexity hidden behind this type of analysis. An overview of the different analytical methods based on DNA measurements and used for the expression of quantitative GM content results is provided, including the use of new techniques based on digital PCR (dPCR).

A measuring system that allows for comparing results by making them traceable to the same reference system has been elaborated in detail. Needs and tools are described and a solution has been proposed to convert results expressing GM content to the required measurement unit, whenever this is needed.

By following these recommendations, results obtained in GM copy number per haploid genome equivalent (cp/HGE) by dPCR can be converted into mass fraction percentage and compared to the results obtained by quantitative PCR (qPCR) either with a calibrant certified for its GM mass fraction or with a calibrant certified for its GM purity.

The general principle is to relate a measurement result to a GM quantity embedded in a specified certified reference material (CRM) either directly or via one single conversion factor (CF) per event. This conversion factor and its related uncertainty need to be determined precisely for each CRM batch, preferably on the pure GM CRM (100 %), using, for example, dPCR. The estimated uncertainty associated with this conversion factor must be integrated into the measurement uncertainty of the final results expressed in GM mass fraction.

CF are currently not yet established for most CRMs. CF values have been recently reported in a few pioneer dPCR studies. However, such proof of concept studies remain incomplete. Therefore, to avoid a gap between new technologies and current EU regulation, the working group recommends to launch a dedicated study to determine CF values on CRMs. Such a study should involve a limited number of competent laboratories with a proven experience in dPCR. The study could be coordinated by the EURL-GMFF.

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3 Glossary

AOCS	American Oil Chemists' Society
bp	Base pair
cp	Copy
Cq	Quantification cycle
CRM	Certified Reference Material
dPCR	Digital PCR
EC	European Commission
ENGL	European Network of GMO Laboratories
ERM®	Trademark of European Reference Materials
EU	European Union
EURL GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
gDNA	Genomic DNA
GM(O)	Genetically Modified (Organism)
HGE	Haploid Genome Equivalent
ISO	International Organization for Standardization
ISTA	International Seed Testing Association
JRC	Joint Research Centre
LLP	Low Level Presence
LOQ	Limit of Quantification
m/m	Mass to mass ratio (mass fraction)
MPR	Minimum Performance Requirements
MU	Measurement Uncertainty
N	Number of samples
n	Number of measurements on the same sample
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
pDNA	Plasmid DNA
qPCR	Quantitative (real-time) PCR
QA	Quality Assurance
QC	Quality Control
SI	International System of units

4 Scope

This document should guide the conversion of analytical results expressed in GM copy number per haploid genome equivalent (cp/HGE) into results expressed in GM mass fraction (and vice versa). The conversion would allow comparability and traceability of the results in cases where such conversion is needed for analytical or legislative reasons.

There are several analytical techniques for the identification and quantification of the GMO presence in food, feed and seeds. This document is related to quantitative DNA-based methods such as qPCR which are the accepted methods for the quantification of GMOs in the EU.

The document also considers digital PCR (dPCR), which is presently not (yet) used routinely for GMO analysis by control laboratories. Protein-based methods are only mentioned briefly and whole genome sequencing is not discussed here.

A recommended strategy and the factors to be used for the conversion of GMO quantification results between cp/HGE and mass fraction are provided in this document.

The document outlines how comparable and metrologically traceable results can be established from qPCR or dPCR measurements, if the data are anchored to the certified value of a reference material.

5 Definitions

For the purpose of this document, the definitions in Regulation (EC) No 1829/2003 apply **[1]**. 'Genetically modified organism' or 'GMO' means a genetically modified organism as defined in Article 2(2) of Directive 2001/18/EC **[2]**, i.e. 'genetically modified organism' means "*an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; Within the terms of this definition: (a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1; (b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification*". 'Genetically modified food' means "*food containing, consisting of or produced from GMOs*". 'Genetically modified feed' means "*feed containing, consisting of or produced from GMOs"*.

Haploid Genome Equivalent (HGE) *A haploid genome corresponds to a single complete set of chromosomes transmitted vertically via maternal or paternal germ cells (ovule or pollen, respectively). Each HGE will contain one or more genetic markers that can be used as target for identification and quantitation of the species as well as one or more genetic markers that can be used for identification and quantitation of the GMO derived DNA.*

Metrological traceability is internationally defined as the 'property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty'.

6 EU legislation

This chapter summarises the description of the unit of measurement for GMO analysis as defined in EU legislation.

6.1 Directive 2000/13/EC (Article 8) and Regulation (EU) No 1169/2011 (Article 23)

Provisions on the measurement unit to be used to express the net quantity of pre-packaged foodstuffs were set out in Article 8 of the EU Directive 2000/13/EC **[3]** and further adopted in Article 23 of the EU Regulation 1169/2011 **[4]**. This Regulation of the European Parliament and of the Council on the approximation of the laws of the Member States concerns the labelling, presentation and advertising of foodstuffs. The purpose of this Regulation is to enact community rules of a general nature applicable horizontally to all foodstuffs on the market. The prime consideration for any rules on the labelling of foodstuffs is the need to inform and protect the consumer. Article 23 provides that the net quantity of pre-packaged foodstuffs shall be expressed *in unit of mass* in the case of "products" that are not liquids, using kilogram or gram as appropriate. The Regulation does not address specifically GMOs but it is a horizontal regulation applying to all foodstuffs, including, in the absence of specific provisions, GM food. This Regulation is valid for food but does not apply to feed.

6.2 Directive 2001/18/EC

The Directive 2001/18/EC **[2]** concerning the deliberate release of GMOs into the environment, and repealing Council Directive 90/220/EC, defines in Article 2 that "*product*" means a preparation consisting of, or containing, a GMO or a combination of GMOs, which is placed on the market. The precise additional information on the nature of the genetic modification for the purpose of placing GMOs on the market is defined in ANNEX IV-A-7 and includes the methodology needed to detect and identify GMO products. However, there is no reference to a specific unit of measurement mentioned.

6.3 Regulation (EC) No 1829/2003

Regulation (EC) No 1829/2003 **[5]** outlines the principles for, and regulates the placing on the market of food and feed consisting of, containing or produced from GMOs. It provides the general framework for the regulation of GM food and feed in the EU and establishes the JRC as the European Union Reference Laboratory for GM Food and Feed. Articles 12 and 24, defining the scope of the regulation as regards labelling explain that labelling "shall not apply to food/feed containing material which contains, consists of or is produced from GMOs in a proportion not higher than *0.9 per cent* of the food/feed and of each food/feed of which it is composed, provided that this presence is adventitious or technically unavoidable". The percentage mentioned here is not *per se* a unit of measurement as it does not explain to what the percentage refers.

6.4 Regulation (EC) No 1830/2003

Regulation (EC) No 1830/2003 [6] defines the traceability and labelling provisions for GMOs and the traceability requirements of food and feed products produced from GMOs, which have been authorised under Directive 2001/18/EC (part C) or under Regulation (EC) No 1829/2003. The traceability mentioned here is not the metrological traceability of a measurement result but is understood as the ability to track GMOs and products produced from GMOs at all stages of their placing on the market. Similarly, as in Regulation (EC) No 1829/2003, a percentage is provided as the threshold for adventitious or technically unavoidable presence but the unit of measurement is not defined.

6.5 Regulation (EC) No 641/2004

Regulation (EC) No 641/2004 [7] provides detailed rules for the implementation of Regulation (EC) No 1829/2003, including method validation (Annex 1) and reference materials (Annex 2). It applies to food and feed containing, consisting or produced from GMOs other than GM plants to which Regulation (EU) No 503/2013 applies. Annex 1 makes it clear that polymerase chain reaction (PCR), and, for example, real-time PCR needs to be used to quantify GM food/feed and refers to the ENGL guidance method acceptance and method performance criteria (that has been revised in 2015 [8]). Annex 2 defines a certified reference material and how the procedure used to establish the property value of a CRM makes this value traceable (in a metrological way) to an accurate realisation of the unit in which the property value is expressed. This means that the result obtained by a qPCR method calibrated with a CRM should be expressed in the measurement unit in which the property value of the GMO CRM is certified.

6.6 Regulation (EC) No 882/2004

Regulation (EC) No 882/2004 [9] lays down general rules on official controls performed to ensure the verification of compliance with feed and food law. It establishes the European Union and National Reference Laboratories (EURL and NRLs) and lists their tasks. It designates the existing EURL for GM food and feed (according to Regulation (EC) No 1829/2003) as EURL for GMO. Chapter III mentions that Competent Authorities should designate official control laboratories and these should operate according to and be accredited to ISO/IEC 17025 standard. Annex III lists the criteria for the assessment of a method of analysis but does not define a unit of measurement.

6.7 Recommendation 2004/787/EC

The EC Recommendation 2004/787/EC [10] gives technical guidance for the sampling and detection of GMOs and material produced from GMOs (food, feed and seeds) in the context of Regulation (EC) No 1830/2003. The Commission recommends in Chapter 4 entitled "analytical testing" and in Chapter 6 entitled "expression and interpretation of the results of the analyses" that results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes.

6.8 Regulation (EU) No 619/2011

Regulation (EU) No 619/2011 [11] applies to feed as regards the presence of GM material for

which an authorisation procedure is pending or the authorisation has expired. This is the latest Regulation, which gives technical guidance on the expression of GM results. The technical content of this Regulation concerns methods of sampling and analysis and is similar to the earlier EC Recommendation 2004/787/EC. However, the expression of the result of GM analysis must be in mass fraction. In addition, Annex II on "criteria for sample preparation and methods of analysis", states that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF".

Moreover, Regulation (EU) No 619/2011 (Article 3) states that "the certified value of the GMO content shall be given in mass fraction and, where available, in copy number per haploid genome equivalent".

6.9 Regulation (EU) No 503/2013

Regulation (EU) No 503/2013 **[12]** implements Regulation (EC) No 1829/2003 and amends Regulation (EC) No 641/2004 by, inter alia, providing specific requirements in Annex III regarding the performance characteristics of the submitted method(s), technical requirements regarding the type of information that the applicant must provide so as to verify that those requirements are met, regarding samples of the food and feed and their control samples, as well as the certified reference material. Note that the scope of this Regulation is restricted to GM plants and derived food and feed only, while for GMOs other than plants, Regulation (EC) No 641/2004 applies.

It is mentioned that the certified value of the GMO content in a CRM shall be given in mass fraction and, where available, in copy number per haploid genome equivalent.

Paragraph 3.3 of Annex III indicates that the applicant may use the same raw material for the production of the certified reference material and for the production of control samples. Consequently, a raw material with a certain zygosity could be used for the validation of a method for a GM-specific event whereas another raw material with a different zygosity may be used for the preparation of a CRM. Therefore, the information about the zygosity that is provided in each validation report by the EURL GMFF does not systematically allow converting results expressed in copy number per haploid genome into mass fraction as stated in Regulation (EU) No 619/2011 (see also 8.1). This guidance document proposes a pragmatic and scientific approach to solving this issue.

7 Feed law regarding botanical impurities of GM origin

In the case of botanical impurities, the GMO content is expressed in a different way than for GM food and feed products. The GM content of botanical impurities in feed samples is reported as the mass fraction of the GM species towards the whole mass of the feed material rather than towards the species of impurity.

A botanical impurity is an impurity that was not intended to be present in a feed material. In such a feed material, e.g. oilseed rape cake, the presence of a non-toxic impurity, e.g. soybean cake, is considered negligible. The feed material is reputed as pure as long as the level of

impurity does not exceed a certain threshold (mostly 50 g/kg). However, it may vary according to the feed material and those feed materials derogating to this general rule are indicated in the list of feed materials of Part C of the Annex of Regulation 68/2013 **[13]**. The issues become more complex when the botanical impurity is completely composed of or partially composed of GM species (authorised in the EU) as this raises the question of whether labelling is required or not. A GM botanical impurity is often composed of pure GM plant material for the species considered (i.e. content close to 100 % in mass fraction towards the plant species of that material). It is agreed (SCFCAH, 2004 **[14]**, confirmed by the European Commission, 2006 **[15]**) that in such a case the only valid way to express the content of genetically modified material of a botanical impurity in the feed material is not in mass fraction towards the species of the material of the contaminant but towards the whole mass of the feed material.

A practical example can illustrate that: consider an oilseed rape oil cake in which soya bean material is present as a botanical impurity at a concentration of 10 g soya bean per kg of cake [1 %] and that this material is genetically modified at 800 g GM soya bean per kg soya bean. According to the rules of the regulation 1829/2003 in which the result is calculated per ingredient (species), the GM soya bean content in soya bean would thus be 80 % (m/m), but as botanical impurity, the GM concentration would be calculated as 1 % multiplied by 0.8 which ends up in 0.8 % (8 g of GM soya bean per kg of cake). As this level is below 0.9 %, no labelling is required. If the amount of soya bean in the cake would reached 2 % (20 g soya bean per kg cake), then the final amount of genetically modified material should be calculated as 2 % multiplied by 0.8 which is 1.6 %, hence, requiring GM labelling.

However, this approach for expressing the GM content has some drawbacks:

1) The measurement of the content of genetically modified material towards the whole feed material is not straightforward. The qPCR approach based on a ratio of data from simple DNA fragment measurements will only provide the content towards the plant species under consideration (note that the same holds true for dPCR). Therefore, another approach such as light microscopy is required to determine the level of the botanical impurity in the feed material to ensure that it does not exceed the threshold set for a botanical impurity.

2) Although a feed material consists in most cases of a single plant species which facilitates the detection of botanical impurities, there are cases where a feed material is composed of several plant species (e.g. when biscuits are recycled in feed). Then the detection of contaminants may be more difficult if the contaminating plant species is also part of the feed material.

8 Metrological traceability and reference systems

Metrological traceability is internationally defined as the 'property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty' **[16]**. This definition already emphasises that measurement results without a measurement unit cannot be traceable. Note that a result without an appropriate uncertainty statement is also not traceable.

The chain of calibrations establishes the so-called traceability chain. Traceability is a property that makes measurement results meaningful and comparable. It is therefore included in quality standards such as ISO/IEC 17025 **[17]** and essential for enabling trade, labelling thresholds, quality control, etc.

Besides the measurement unit, there are different types of references, i.e. anchor points, for setting up metrological traceability. The reference can be a measurement procedure. Here, the procedure defines what is measured (operationally defined measurand). This is often described in a documentary standard or by a reference method. Another option is that the measurand is defined independently from a method or methodology. In both cases, the property value embedded in a Certified Reference Material (CRM) can be used to establish and control the traceability chain. The measurement result is made traceable to the certified value of the CRM. In relation to GMO quantification the metrological traceability of a measurement result is ensured by setting up an arbitrary reference system composed of a reference method (the PCR method validated by the EURL GMFF) and the CRM. Reference method and certified value of the CRM are used together to ensure comparability of measurement results. Unfortunately, the situation in GMO quantification is not ideal, as no independent quality control and calibration materials exist in many cases. However, the measurement system set up for GMO quantification does not necessarily require this.

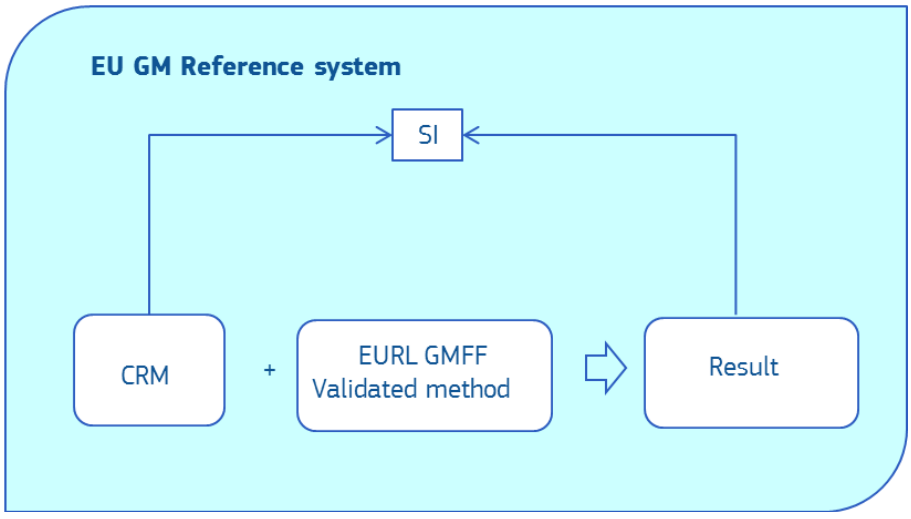

Figure 1: The reference system used to quantify a GM content in the EU is composed of a series of validated methods (one per GM event) that in combination with a series of certified reference materials (one per GM event) gives a defined result. As the CRM value used is traceable to the International System of units (SI), the result is also traceable to the SI. The unit of measurement of the result is the unit in which the CRM value is certified.

The traceability chain for the property value of a CRM certified for its mass fraction is based on the use of calibrated balances and a thorough control of the weighing procedure. The certified value is therefore traceable to the International System of Units (SI). Such CRMs are intended to be used as a calibrant for qPCR measurements of a particular GM event in food, feed and seed. Consequently, these CRMs are establishing, together with the measurement method validated by the EURL GMFF, a reference system required for quantification of a particular GM event.

A solution is provided in this guidance document for linking a result that has been expressed in cp/HGE, e.g. resulting from applying dPCR, to a quantity value embedded in a CRM, using a specific conversion factor thereby providing traceability to the CRM's property value.

9 Available tools and guidance documents

9.1 EURL GMFF validated methods

The EURL GMFF is tasked by Regulation (EC) No 1829/2003 to evaluate and validate methods for detection, identification and quantification of GMOs. It is also mandated for receiving control samples for use during the method validation procedure and for distributing appropriate control samples (not necessarily the same as those used for method validation) to the NRLs. In this context, the material supplied to the EURL GMFF by the applicant under the provisions of the Regulation is legally defined [18] as follows: "control sample" means the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample). As part of the authorisation procedure for a new GMO, the applicant, therefore, submits usually DNA of the GMO and of its isogenic (non-GMO) parental line to the EURL GMFF. Following the guidance provided to applicants [19], the positive control sample must contain 100 % GMO and the negative control sample must be the exact conventional counterpart. This DNA is then used by the EURL GMFF to validate the analytical method developed by the applicant for the specific GM event.

Following EC Recommendation 787/2004/EC and the advice of the ENGL, and given that the production of CRMs is usually still ongoing when the validation exercise has to start, the EURL GMFF usually adopted the GM copy number ratio as unit of measurement in the context of collaborative trials to validate the event-specific qPCR method for a new GMO DNA.

In determining the GM copy number ratio in a DNA sample, the number of copies of the GM event sequence, as well as the number of copies of a species- or taxon-specific endogenous sequence, must be determined. To do so, the zygosity of the positive control sample, as well as other biological factors (see Section 9.4), must be known. Since 2011, the EURL GMFF therefore, verifies the zygosity of the positive control sample by dPCR as described in the validation reports [20]. The validation reports therefore specify an event-specific conversion factor that is used to prepare the standards and test samples used during method validation. Regulation (EC) No 619/2011 states that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF".

Despite this statement, it is scientifically not correct to use the conversion factor experimentally estimated on the GMO control samples for the measurements performed by control laboratories (if needed). In practice there may not be a large difference between the conversion factor determined on the GMO control sample and the CRM, as both are often derived from similar plant materials provided to both the EURL GMFF and a CRM producer. However, this is not always the case, and sometimes the conversion factor determined during the validation (on homozygous maize) does not correspond to the one applicable to the CRM (produced from hemizygous maize). Also in case a taxon-specific reference target is used in the control laboratory different from the one used to establish the zygosity during method validation, the conversion factor mentioned in the validation report may not be applicable anymore as some of the endogenous targets are present in two or more copies in the genome. It is clear that the metrological traceability is guaranteed only if the conversion factor is determined on a material with a certified property value such as a CRM.

9.2 Certified reference materials used for calibration

Quantification of the GM content in food or feed products relies mainly on the detection, amplification and relative quantification of well-defined DNA sequences. The relative quantification is providing a DNA fragment ratio arising from relating the measured amount of a specified DNA sequence to the measured amount of another DNA sequence. It should be kept in mind that qPCR needs to be calibrated to convert a measured fluorescence signal into a quantity characteristic for the amount or mass of the DNA fragment of interest. The "kind" of this quantity is intrinsically determined by the certified property of the calibrant used. Consequently, the unit of measurement associated with a result is also determined by the unit of measurement in which the calibrant has been certified (Table 1).

Table 1: Nature of different CRMs used as calibrant and the unit of measurement of the certified property value.

Type of CRM	Matrix	Unit(s) of measurement of the certified value	
Pure seeds	Seeds, GM or non-GM	g/kg	-
Pure powders	Milled seeds, GM or non-GM	g/kg	-
Mixed powders	Milled seeds, GM and non-GM	g/kg	cp/HGE
DNA extracts	DNA extracted from plant leaves, only GM	ND[1]	-
plasmid DNA	Dual-target plasmid, containing GM and non-GM targets	-	cp/HGE

Four different types of CRMs could be available to calibrate a qPCR method. The CRMs could be pure milled GM seeds or intact GM seeds, certified to contain at least a certain mass of GM material per kg of total material. The CRMs could also be a mixture of milled GM seeds and milled non-GM seeds certified to contain a certain mass fraction of GM material in the total mass; a limited number of these materials are additionally certified for the copy number ratio between GM DNA fragments and taxon-specific DNA fragments. In some cases (e.g. AOCS), leaf material (more uniform tissue with respect to zygosity) has been used to prepare DNA CRMs certified for GM event presence or purity. A few dual target plasmids containing a single copy of both the GM event and the taxon-specific target have also been certified. These plasmid DNA (pDNA) solutions can be used to calibrate qPCR experiments. However, they constitute a different reference system than extracted genomic DNA. Indeed, despite the fact that the commutability of pDNA has been demonstrated for some GM assays [21], small differences in PCR efficiencies have been observed for gDNA and pDNA for other GM assays, which means that the result is traceable to the particular calibrant used [22].

[1] ND: not defined. CRMs sold by AOCS are certified for the presence of a GM event, specifying "pure" homozygous or heterozygous GM event. The material is considered as 100 % or 1000 g/kg.

The measurement unit which is used for the certified value of the calibrant used to calibrate a qPCR analysis determines the measurement unit of the analytical result. If the calibrant is a CRM certified for its mass fraction, the results must be expressed in mass fraction. If the calibrant is certified for its DNA copy number ratio, then the results must be expressed as a ratio of DNA copy numbers; in line with Regulation (EU) No 619/2011, the latter results should be converted to a GM mass fraction by applying a conversion factor.

9.3 Other guidance documents

An earlier EURL GMFF technical guidance document on the implications of Regulation (EU) No 619/2011 for feed analysis **[23]** states the following: "When results are expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, they shall be either re-analysed with an appropriate calibrant for mass fraction measurements or converted into mass fractions by taking the associated additional uncertainty into consideration". The CRMs frequently used for calibration are certified for their GM mass fraction; use of such calibrants leads to measurement results expressed in GM mass fraction (i.e. m/m %), without the need for any conversions. For homozygous GM varieties (e.g. GM soya bean), the results remain equal whether expressed in GM mass fraction or GM copy number ratio. For hemizygous GM varieties (most GM hybrid maizes), assuming the (plasmid) calibrant used was certified in GM copy number ratio, the document suggested a simplified approach, namely to multiply the measured GM maize copy number ratio by 2 to obtain the result in GM mass fraction. The factor 2 was chosen as a first approximation, knowing that most maize commodities are derived from hybrid maize hemizygous for the GM event and assuming the equal frequency of male and female parental GM-contributions in these commodities.

As explained in the document, this simplified approach cannot guaranty equivalence and traceability of results expressed in both units of measurements among laboratories using different calibrants or laboratories performing their analysis by dPCR. It is, therefore, important that the reference system defined by the method and the CRM is also applied here. The factor 2 can be used as a first approximation; however, for the comparability of measurement results over a longer time perspective, the conversion factor has to be established on the specific GMO CRM basis.

9.4 Genetic constitution of seeds

Biological factors related to the tissue types and genetics of the plants do not affect the DNA-based GM quantification when an arbitrary reference system is used that as proposed in this document (Section 13).

When considering the effect of seed biology on GM quantification without using a reference system, one should bear in mind that food and feed products may be produced from whole seeds or from specific seed parts, which considerably complicates the situation as each of the seed parts have a different composition (also concerning their GM content). Examples are maize germ (embryo), maize flour (from seed endosperm), soybean hulls (from seed coat), oilseed rape oil (from seed endosperm and embryo), press-cake (whole seeds after pressing out the oil), etc. During plant development, the seeds are formed by the fusion of reproductive cells from the female and the male parent, followed by specific processes of cell division, resulting in the formation of different tissues within the seed and with specific ploidy levels and in some cases with a different genetic constitution. Moreover, monocot and dicot plants have substantial differences in seed development, morphology and genetic composition:

- Monocot seeds are made up of a diploid seed coat, originating from the wall of the embryo sac (mother tissue), a triploid endosperm, containing two sets of chromosomes from the mother and one set from the father, and a diploid embryo, containing one set of chromosomes from the mother and one set from the father.

The relative total amount of DNA found in these three tissue types is variable between species and also depends on the variety.

- Dicot seeds are made up of a diploid seed coat, originating from the wall of the embryo sac (mother tissue), a diploid endosperm, which has stopped to divide, and a diploid embryo. Both endosperm and embryo contain one set of chromosomes from the mother and one from the father.

The GM seeds can be homo- or hemizygous relative to the GM event. The zygosity of the seeds will to a large extent depend on the breeding practices for the variety, the type of pollination (self- or cross-pollination), the parental origin of the GM event (from the female or male parent), and to a lesser extent on the origin of the seeds, i.e. from an agricultural field on which the GM plants were planted or resulting from outcrossing into a neighbouring non-GMO field.

Seeds are either homozygous, such as in soya bean or hemizygous, such as in maize, which is almost only cultivated as a hybrid crop. GM soya bean seeds are (currently) produced by self-pollination and selection of homozygous transgenic progeny. Outcrossing rates are very low; therefore, the chance of hemizygous seeds to be formed under natural conditions is very low. Maize seeds, on the contrary, are produced by crossing two selected lines to obtain hybrid seeds which, when cultivated, display the expected hybrid vigour. Most maize hybrids are obtained by crossing a female GM line with a male non-GM line (even for GM stacks), although exceptions exist. The maize female and male flowers are physically separated and the male flowers of the GM line are therefore usually removed (mechanically or chemically) for producing the hybrid seeds.

Grains (or kernels) used for food and feed production consist of the progeny of the GM plants and can be the result of self-pollination or cross-pollination:

- Soya bean has closed flowers and is a typical example of a self-pollinating species. As GM soya bean plants are homozygous; also the harvested beans from the species will be almost entirely homozygous for the GM event(s).

- Maize is a typical cross-pollinated crop, although self-pollination may also occur. As nearly all maize seeds are hemizygous, the progeny will be a mixture of non-GM, hemizygous GM and homozygous GM kernels in a ratio 1:2:1. While the CRMs produced so far are from nearly pure hybrid seed (sowing seed), the kernels used in food and feed are a heterogeneous mixture (progeny seed), which on average is composed of 75 m/m % GM kernels (1/3 homozygous, 2/3 hemizygous).

- Oilseed rape is mostly self-pollinating, given that the male and female organs are close to each other within the flower. However, under natural conditions, outcrossing also occurs (from 5 to 30 % **[24]**). The traditional breeding practices for this species result in the production of homozygous GM seeds. More recently, however, increasing attempts have been done to produce hybrid oilseed rape varieties. While most progeny seeds used for food and feed will, therefore, be homozygous, the market situation may change when hybrid varieties will increasingly be used. On the other hand, oilseed rape is mainly used for oil production, in which DNA is hard to detect. However, whole oilseed rape seeds and oilseed rape cake may be used as high-protein animal feed.

Several studies in the last ten years have provided information on the influence of the parental origin of the transgene on the DNA-based quantification [25, 26, 27, 28]. Maize was used as a case study because the maize kernel is a good model system to study the DNA content in different tissues, the number of maternal and paternal genome copies in the different tissues and their GM content, and to model the influence of these factors on the DNA-based GM quantification [29] (Figure 2).

The studies on the DNA content and distribution of the transgenic alleles in seeds demonstrated a considerable difference between the GM-content determined in seed number, genome percentage or mass percentage. Several factors contribute to this discrepancy, including the use of specific seed tissues for food and feed production (maize embryos as high protein feed, maize endosperm for flour, etc.), varietal differences in the relative mass fraction of the different seed tissues and their DNA content, the parental origin of the GM event, and the maturity of the seed (which differs considerably between food and feed production).

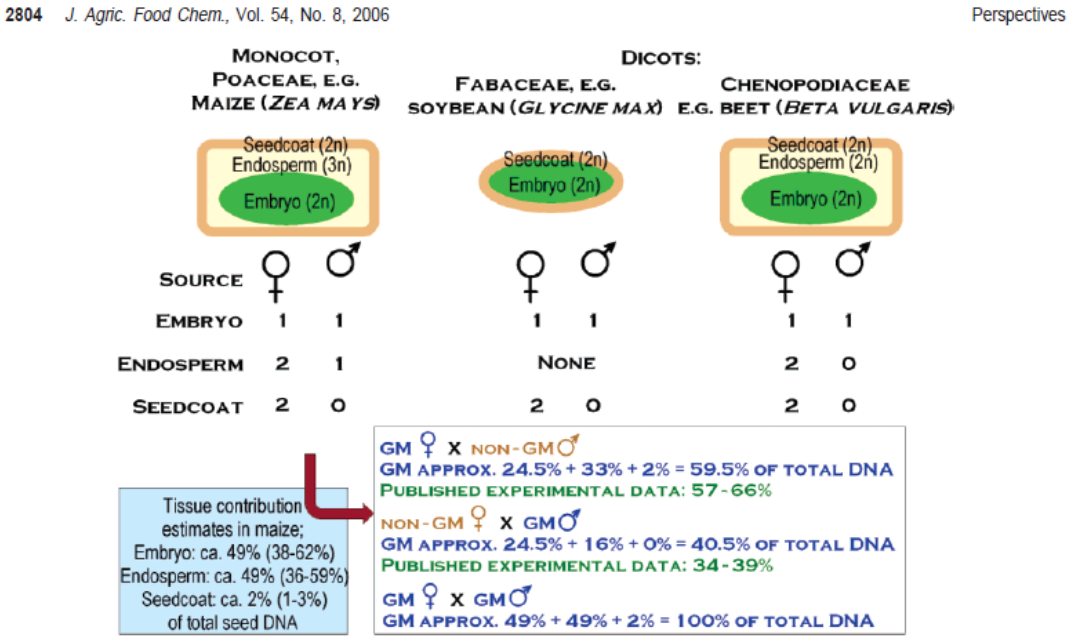

Figure 2: Contribution of haploid genomes from the parental gametes in plant seeds. The genetic influence of the parents on the DNA-based GMO content is exemplified with maize based on extrapolation from data published in the literature. The beet seed does not have an endosperm, but instead has a strictly maternal perisperm with endosperm function. Additionally, the sugar beet seed has a maternally inherited pericarp (Figure from Holst-Jensen *et al.* 2006) [29].

Following the above considerations, it is clear that, except for intact seeds or flour made from intact seeds, the zygosity status of the GMO loci in an unknown sample cannot be determined precisely.

As a consequence, the use of a reference system defined by a CRM and an EURL-validated method, together with a defined conversion factor based on the GM CRM referenced for each EU-authorised event is the best way to circumvent all the problems related to the biology and genetics of (hemizygous) GM seeds.

10 DNA and protein-based analytical methods

This chapter will mainly cover DNA and protein-based methods used to quantify GM materials.

10.1 DNA-based methods

qPCR is the most established quantification method currently applied for GM quantification; however some research and control laboratories have started to use dPCR as an alternative method for qPCR. Both analytical methods are considered in this guidance document.

10.1.1 Quantitative PCR (qPCR)

qPCR requires the use of a calibrant in each analysis to compare the fluorescence signal of a sample to the signal obtained with a certified reference material. The unit of measurement depends on the unit of measurement of the certified value of the calibrant (see Section 9.2). Reference materials can be certified using either m/m or cp/HGE. In case the CRM is certified for the degree of (GM or non-GM) purity, the purity is expressed as a mass fraction (expressed in g/kg).

10.1.2 Digital PCR (dPCR)

dPCR enables quantification of the number of targets present in a sample, using limiting dilutions, PCR and Poisson statistics. The PCR mix is distributed across a large number of partitions or droplets containing zero, one or more copies of the target nucleic acid. After end-point PCR amplification, each partition is scrutinised and defined as positive (“1”, the presence of PCR product) or negative (“0”, the absence of PCR product) hence the term “digital”. The absolute number of target nucleic acid molecules contained in the original sample before partitioning can be calculated directly from the ratio of the number of positive to total partitions, using Poisson statistics.

Two types of partitioning are currently used in commercially available dPCR systems. In chamber digital PCR (cdPCR), the partitioning of up to a few thousand individual reactions is done in microfluidic chambers. In droplet digital PCR (ddPCR), the partitioning of the PCR assay is realised into several thousands or millions of individual droplets in a water-oil emulsion. The fluorescence of the droplets is measured by flow cytometry for counting the PCR positive and negative droplets.

A few research groups have verified the applicability of dPCR for GMO testing **[30, 31, 32]** but the technique has not (yet) been implemented as a routine method in official laboratories for GMO testing. dPCR enables detection of a single target copy and is an endpoint measurement less prone to suboptimal PCR efficiencies. dPCR does not measure the number of PCR cycles needed to reach a fluorescence threshold, and a calibration solution with a known amount of copies of the targeted DNA is not needed. For GMO testing, the number of positive partitions (or droplets) and their total numbers for the reference DNA target and the transgenic DNA target are counted and the ratio of the copy number is calculated to estimate the GM percentage expressed as copy numbers per HGE.

10.2 Protein-based methods

In the EU, protein-based methods such as ELISA immuno-strip technology (lateral flow strip, dipstick) only play a role in seed testing. For other purposes, protein-based methods are not commonly used for several reasons: lack of event specificity, less sensitivity, high uncertainty and low applicability to processed materials. For ELISA, a calibration curve needs to be established using a matrix material which is often certified for its mass fraction content. As a consequence, GM quantities are expressed as a mass fraction (g/kg) traceable to the CRM used and do not need to be converted.

11 Seed testing

The detection of genetically modified seeds in seed lots depends on different assays, based on DNA, protein or the plant phenotype (bioassays). According to the Commission Recommendation 2004/787/EC, DNA-based (event-specific) assays are the recommended for the analysis of seeds or other propagating materials.

However, all three methodologies are considered by the International Seed Testing Association (ISTA). Chapter 19 of the ISTA Rules **[33]** is provided as a guideline for testing the adventitious presence of GM seeds as well as for GMO trait purity testing in seed lots. According to the Performance Based Approach (PBA), adopted by ISTA, the laboratory can use any test method, as long as it has been validated and the laboratory complies with given performance standards. To cope at the international level with different aims and situations, ISTA accepts the expression of results in three different units of measurements:

- as % in mass of seeds, used when a standard curve is prepared using reference materials certified for GM mass fraction (g/kg).
- as % of DNA copies, used when a standard curve is prepared using reference materials certified for GM copy number ratio (cp/HGE).
- as % in number of seeds, used to estimate the percentage of GM seeds in the seed lot. It is applicable both to single seed testing and in case a subsampling approach is adopted. To obtain the results in percentage of the number of seeds, ISTA provides SeedCalc software **[34]**, a free statistical tool that can be used to design seed testing plans, including those addressed to estimate the adventitious presence levels of biotech traits in conventional seed lots. A new version of the ISTA software - SeedcalcStack version 9 **[35]** can be used for the estimation of the proportion of GM seeds containing up to three stacked events in a conventional seed lot.
  For the expression of the GM content as percentage of number of seeds, a statistical sampling plan in combination with a qualitative assay can be used. It estimates if the GM content in a seed lot is above or below a specified value with a certain confidence level. The result can be calculated using ISTA SeedCalc or the statistical tool described in the report of the Working Group for Seed Testing **[36]**.

These three units of measurement are considered by ISTA to cope with different needs, but no information is provided to compare the results expressed in the different measurement units.

Taking into account the different testing approaches and the biological factors previously described, the conversion between GM seed percentage expressed as a number and the two other units being either mass fraction or copy number ratio is not feasible with acceptable uncertainty.

12 Analytical results and assumptions made

12.1 Analytical results reported in mass fraction

When CRMs certified for their GM mass fraction (g/kg) are used as calibrant, DNA needs to be extracted from each CRM of the CRM series or from the CRM with the highest concentration, and likewise from the samples to be analysed, using either a DNA extraction method that has been validated by the EURL GMFF and in-house verified or by an in-house validated DNA extraction method. The total concentration of extracted DNA can be quantified either by a UV spectrophotometric method using the appropriate molar absorption coefficient [37], or by a fluorometric method such as the PicoGreen® Assay for double stranded DNA or any other preferred method. The extracted DNA concentration is measured to determine the volume of DNA solution needed for the PCR assays targeting the GM-DNA and the reference gene, respectively. It is important to know which amount of DNA has been added in both PCR assays but as the measurement is based on a ratio, a high precision of the DNA concentration is not required.

Once the total DNA concentration has been determined for the reference material and for the samples to be analysed, two calibration curves are made by plotting the number of PCR cycles needed to reach a certain fluorescence level (Cq values) against the logarithm of the amount of DNA in the PCR. The Cq value for the unknown sample is measured and that value is used to calculate the amount of DNA target present in the unknown sample. The slope and the coefficient of determination are also calculated to verify that the PCR assays are fulfilling the minimum acceptance criteria defined by the ENGL [38].

Example: the DNA in the PCR assay targeting the reference gene is diluted in buffer or nuclease free water (e.g., dilutions from 150 ng DNA/PCR to 1 ng DNA/PCR are used to establish a calibration curve for the reference gene). To generate a calibration curve for the GM-DNA target, 150 ng DNA/PCR extracted from CRMs containing a decreasing amount of the GMO (e.g., from 50 g/kg to 1 g/kg) are used. The amount of GM DNA in the assay that has been extracted from a CRM containing 50 g/kg GM (corresponding to 5 m/m %) is considered to be also 5 % in terms of GM DNA copies (i.e. 7.5 ng of GM DNA per PCR well for a reaction containing 150 ng DNA). The same proportional approximation is made for the other standards that contain a smaller mass fraction of GM or reference gene targets. The amounts (or concentrations in %) of GM-target and reference gene DNA in a DNA solution extracted from an unknown sample are then calculated by converting the measured Cq values into mass values using the two calibration curves and dividing them. The GM mass fraction (GM target versus reference gene target) is finally multiplied by 100 to express it as a percentage.

In this process, two assumptions are made: the PCR efficiencies for the calibrant and the sample are similar and the DNA extraction efficiencies are similar for GM and non-GM material.

12.2 Analytical results reported in copy number per haploid genome equivalent

An analytical result can be reported in cp/HGE when using plasmid calibrant CRMs, or when using a CRM that has been certified for its GM content in cp/HGE, or when applying dPCR. The use of CRMs expressed in DNA copy number ratio for the quantification of GMOs is explained in the ERM Application Note 5 [39].

13 Recommendation

13.1 Conversion of measurement results expressed in mass fraction into cp/HGE

In general, a result expressed in mass fraction does not need to be converted to a DNA copy number ratio to fulfil the current EU legislation. An example of such a conversion of a GM result calibrated with a CRM certified for its mass fraction and converted into copy number is provided in the ERM Application Note 4 [40]. Such a conversion increases the uncertainty associated with the result. Indeed, the uncertainty contributions related to the genome size estimation, to the DNA quantification and the genetic constitution of the seed need to be added to the measurement uncertainty. In the example illustrated in the Application Note 4, the additional uncertainty contributions double the relative expanded uncertainty.
However, with an agreed reference system (method + CRM + conversion factor), the uncertainty related to the conversion from mass fraction into cp/HGE would be the same as the conversion from cp/HGE into mass fraction.

13.2 Conversion of measurement results expressed in cp/HGE into mass fraction

The conversion of a measurement result obtained either by qPCR using a calibrant, expressed as cp/HGE, or by dPCR, into a mass fraction is needed. Different approaches are presented in Figure 3.

The working group recommends anchoring the conversion to the CRM used. In this way a converted result that remains traceable and comparable to a result expressed in mass fraction is obtained. The same result would have been obtained by a qPCR method calibrated with the same CRM. This approach is illustrated in Figure 3 option 1.

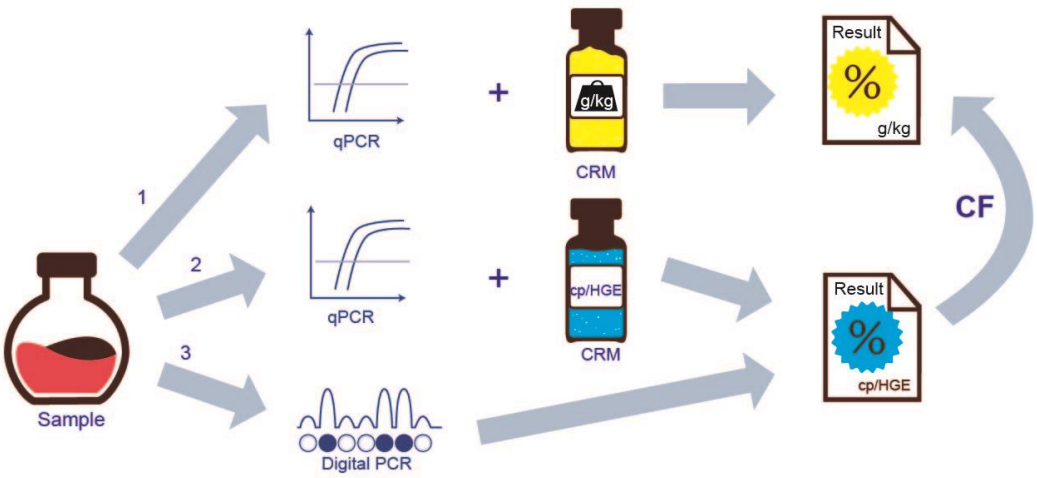

Figure 3: Overview of the various possibilities to measure the GM content present in a sample. In option 1, the GM content is determined by qPCR using a CRM certified for its mass fraction as calibrant. The result is expressed in mass fraction (g/kg). In option 2, the GM content is determined by qPCR using a CRM certified for its copy number ratio as calibrant. The results obtained (in cp/HGE) are converted into a mass fraction (g/kg) using a CF. In option 3, the GM content is determined by dPCR. The result expressed in cp/HGE is converted into a mass fraction (g/kg) by using the same conversion factor (CF).

A pDNA calibrant containing both the reference and the GM-DNA targets in a 1:1 ratio is used to generate two calibration curves. For those calibration curves, a starting solution of the plasmid is diluted and the serial dilutions are used to generate both calibration curves. The measurement unit on the X-axis in a Cq versus log [copy number] plot is the copy number value of the plasmid. This value is provided on the certificate of the CRM as an indicative value. UV spectrophotometry has been used to determine the amount of pDNA copies per µL. This quantity does not need to be determined with a high accuracy provided that the same calibrant solution is used to generate both calibration curves. It is indeed the ratio of the number of GM targets to reference targets which is certified for the CRM, ensuring that the same amount of GM and reference targets are present in both PCR assays when the same solution of calibrant is used. The results obtained using a CRM certified for its DNA copy number ratio can, therefore, be used directly to express the result as a copy number ratio.

Here also two assumptions are made: both DNA targets are amplified with similar efficiency on the dual target plasmid used as calibrant and the PCR efficiencies for the calibrant is similar to the PCR efficiencies of the sample.

Note that the recommended unit of expression for analytical results on GMO quantification is GM mass fraction, not copy number ratio. Therefore, a conversion factor will be needed to convert the values in GM copy number ratio to values in GM mass fraction. The conversion factor to be used in this case could be the same conversion factor as the one based on the CRM provided that the PCR efficiencies for the pDNA are similar to the PCR efficiencies on DNA extracted from the CRM.

A very limited number of (matrix) CRMs have been certified for both their GM mass fraction and GM copy number ratio. Those CRMs can be used as calibrants in the same way as the example provide in chapter 12.1. Despite the fact that the results may be expressed in cp/HGE, such results would anyway need to be converted to mass fraction..

Results obtained by dPCR would need to be converted to mass fraction as well.

This approach is considered as the most elegant way to compare results that would have been obtained using different measurement principles. The copy number ratio remains artificially linked to the CRM.

To perform such an anchoring, the quantity of GM target per haploid genome equivalent in the CRM used needs to be determined and the uncertainty associated with this ratio has to be added to the final measurement uncertainty (after conversion). A few pioneer studies have estimated this ratio by measuring the copy number of GM target versus reference gene target by dPCR in some CRMs [30, 31]. The studies are incomplete and the ratios would need to be verified and validated by interlaboratory comparisons.

Two ways to establish an agreed conversion factor can be identified:

a) Use a unique agreed conversion factor (CF_{sp}) per species independent of the GM assay and independent of the calibrant used. Those CF_{sp} would be based on the market zygosity estimates for particular GM species. As a consequence of different breeding practices (GM target contributed by male or female parent and different DNA extractability from GM and non-GM material) a difference of about 30 % between results that are converted from cp/HGE to mass fraction would need to be tolerated.

b) Use one unique conversion factor (CF_{CRM}) per CRM. A systematic study should be launched to determine for all available CRMs their specific conversion factor. This study would require identifying laboratories with the necessary expertise in dPCR willing to apply an agreed protocol to determine one CF per GM event authorised in the EU. The CF_{CRM} determined by laboratories in interlaboratory comparisons would require official acceptance as part of a measurement procedure and would provide traceability of the measurement results.

The way described in a) does not guarantee a full comparability and traceability between results obtained by qPCR and those obtained by dPCR, but has the advantage of being readily applicable once a conversion factor per species has been agreed upon. This conversion factor is not linked to a particular CRM, but to a theoretical average ratio measured in imported GM commodities.

The way described in b) guarantees this comparability and traceability, but cannot be immediately implemented as the CF_{CRM} need to be determined first.

The uncertainty associated with the conversion factor needs to be added to the uncertainty of the converted results.

It should also be noted that the conversion factor (for the ratio) determined on a 1000 g/kg GM material (or pure GM) will be slightly different from the conversion factor determined from a CRM composed from a mixture of GM and non-GM material for the reasons explained in chapter 9.4. The CF_{CRM} should be ideally determined on the pure GM CRM, however such CRMs are not always available.

Once a conversion factor is agreed or has been determined, the conversion of results expressed as a ratio of copy numbers into mass fraction can be done using the equation (1).

$$GM\ \%\ \left(\frac{m}{m}\right) = \frac{copy\ number\ of\ the\ GM\ specific\ sequence}{copy\ number\ of\ the\ taxon\ specific\ sequence}\ \text{x}\ \frac{1}{CF}\ \text{x}\ 100 \qquad (1)$$

The uncertainty (u_{CF}) of the conversion factor CF should be combined with the measurement uncertainty (u_m) using equation (2) to calculate the expanded uncertainty (U) associated to the GM results (*k* = coverage factor).

$$U = k.\sqrt{u_m^2 + u_{CF}^2} \qquad (2)$$

The use of conversion factors to convert copy number ratio into mass fraction is not new and has been applied in Japan [**41**]. Conversion factors have been proposed as part of a MON810 quantification method in the informative (not normative) Annex C5 of ISO 21570 [**42**]. In that method, a plasmid RM has been used to measure the ratio between MON810 specific targets and taxon-specific *zSSIIB* (starch synthase IIb gene*)* targets in DNA extracted from one particular MON810 seed line. DNA extracted from an unknown sample is then analysed by qPCR using the reference plasmid as calibrant. The result obtained expressed as copy number ratio is then converted into mass fraction by dividing the results by the CF.

The conversion of a copy number ratio into mass fraction presented in this guidance document is similar to the approach followed in Japan. In both cases CFs are determined per GM event and not per GM species. However, the technique used (dPCR) as well as the material (CRMs) chosen to determine the CFs are different.

13.3 Example

The practical implementation of the conversion factor in real life is illustrated by the following example. A fictitious sample to be analysed would consist of maize and soya. The identification revealed that three soya GM events (MON-Ø4Ø32-6, MON-89788-1 and DP-3Ø5423-1) and two maize GM events (MON810 and MON-ØØ6Ø3-6) are present in the sample. For reporting the GM content of this sample in mass fraction the experimental data measured by digital PCR in copy number are converted into mass fractions as follows:

GM soya % (m/m) for MON-Ø4Ø32-6 soya = (copy number of MON-Ø4Ø32-6)/(copy number of the taxon specific sequence) x 1/ $CF_{ERM®-BF410}$ x 100

GM soya % (m/m) for MON-89788-1 soya = (copy number of MON-89788-1 soya)/(copy number of the taxon specific sequence) x 1/ $CF_{AOCS\ 0906-B}$ x 100

GM soya % (m/m) for DP-3Ø5423-1 soya = (copy number of DP-3Ø5423-1 soya)/(copy number of the taxon specific sequence) x 1/ $CF_{ERM®-BF426}$ x 100

GM maize % (m/m) for MON-ØØ81Ø-6 maize = (copy number of MON-ØØ81Ø-6 maize)/(copy number of the taxon specific sequence) x 1/ $CF_{ERM®-BF413k}$ x 100

GM maize % (m/m) for MON-ØØ6Ø3-6 = (copy number of MON-ØØ6Ø3-6)/(copy number of the taxon specific sequence) x 1/ $CF_{ERM®-BF415}$ x 100

The conversion of the GM % from cp/HGE into mass fraction is given as example (Table 2). The calculations are made taking into account a relative standard uncertainty reported for the measurement of about 15 % and of about 5 % for the determination of the respective CF_{CRM}. The values of the CF_{CRM} used in this example are fictive. The CRMs listed in this example are the CRMs reported in the Commission decisions authorising the placing on the market of products consisting of, or produced from GM events pursuant to Regulation (EC) No 1829/2003.

The concentrations of GM soya remain unchanged as the respective CF_{CRM} are close to 1. The converted concentrations of GM maize increase as the CF_{CRM} are smaller than 1. The GM maize concentration is not summed but reported per event as it is not possible to differentiate the single events (MON-ØØ81Ø-6 and MON-ØØ6Ø3-6 from a stacked event (MON-ØØ81Ø-6 x MON-ØØ6Ø3-6).

Table 2: Example of the conversion of GM ratio expressed as percentage copy number per haploid genome equivalent into GM percentage expressed as mass fraction using a unique conversion factor per CRM (CF_{CRM}).

	GM ratio (cp/HGE) * U (*k*=2)	% GM (cp/HGE) U (*k*=2)	$u_{m,\ rel}$ (%)	CRM[a]	CF_{CRM}[b] ± u_{CF}	$u_{CF,\ rel}$ (%)	u_{comb} (%)	% GM (m/m) U (*k*=2)
MON-Ø4Ø32-6 soya	0.024 ± 0.006	2.4 ± 0.6	12.5	ERM®-BF410	1.02 ± 0.05	4.90	13.4	2.4 ± 0.7
MON-89788-1 soya	0.006 ± 0.001	0.6 ± 0.1	10.0	AOCS 0906-B	0.98 ± 0.04	4.08	10.8	0.6 ± 0.1
DP-3Ø5423-1 soya	0.014 ± 0.01	1.4 ± 1	17.9	ERM®-BF426	1.00 ± 0.05	5.00	18.5	1.4 ± 0.5
MON-ØØ81Ø-6 maize	0.10 ± 0.03	10 ± 3	15.0	ERM®-BF413k	0.37 ± 0.02	5.41	15.9	27 ± 9
MON-ØØ6Ø3-6 maize	0.09 ± 0.03	9 ± 3	15.6	ERM®-BF415	0.56 ± 0.03	5.36	16.5	16 ± 5

U: expanded uncertainty; *k*: coverage factor; $u_{m,\ rel}$: relative standard measurement uncertainty; CF_{CRM}: conversion factor per CRM; u_{CF} : standard uncertainty associated to the CF; $u_{CF,rel}$: relative standard uncertainty associated to the CF; u_{comb}: combined standard uncertainty (using equation 2); [a]: according to Commission decisions; [b] : fictive conversion factors used as example. * measured by quantitative PCR (dPCR or qPCR).

14 Conclusions

Measurement results expressing a GM content should be reliable, independent of the analytical procedure applied and in line with the EU legislation. The GM "percentage" mentioned in the legislation is not *per se* a unit of measurement as it does not explain to what the percentage refers.
Measurement results obtained by a qPCR method calibrated with a particular CRM should be expressed in the measurement unit in which the property value of the CRM is certified. An attempt was made to clarify the unit of measurement in an EU Recommendation. Namely, it is mentioned that "results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes". However, the most recent EU Regulation specifies that the expression of the result of GM analysis must be in mass fraction. Therefore, GM results that are primarily expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes shall be translated into mass fraction in accordance with the information provided in each validation report of the EURL GMFF. However, it is scientifically not correct to use the conversion factor experimentally estimated on the GMO control samples because that material is not the material used to calibrate the qPCR analysis. Indeed, the metrological traceability is only guaranteed if the conversion factor is determined on a material used as calibrant with a certified property value such as a CRM. The only possibility to have a GM-DNA copy number ratio result traceable and comparable to a result expressed in mass fraction is to determine a conversion factor on the CRM that is mentioned in the Annex of each Commission decision authorising either placing on the market or cultivation of a particular GM event.

To perform such an anchoring, the quantity of GM target per haploid genome equivalent in the CRM used, needs to be determined and the uncertainty associated with this ratio needs to be added to the final measurement uncertainty (after conversion). The preferred option is to use one unique conversion factor (CF_{CRM}) per CRM. This conversion factor and its related uncertainty need to be determined precisely for each CRM batch, preferably on the pure GM CRM (100 %), using, for example, dPCR.

To avoid a gap between new technologies (not requiring an internal calibrant) and current EU regulation, the working group recommends to determine the CF_{CRM} values in a dedicated study. Such a study should involve a limited number of competent laboratories with a proven experience in dPCR. The study could be coordinated by the EURL-GMFF.

In a second step, efforts should be made to have such agreed CF adopted at ISO level to extend comparability of results outside Europe.

Acknowledgments

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